Issued November 28, 1908.

U. S. DEPARTMENT OF AGRICULTURE,

BUREAU OF ANIMAL INDUSTRY.—BULLETIN 109.

A. D. MELVIN, CHIEF OF BUREAU.

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BY

ARTHUR W. DOX,

Chemist in Cheese Investigations, Dairy Division.

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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., September 21, 1908.

Sir: I have the honor to transmit herewith a manuscript entitled "Proteolytic Changes in the Ripening of Camembert Cheese," by Arthur W. Dox, chemist in cheese investigations, Dairy Division, and recommend that it be published as Bulletin 109 of this Bureau. This paper deals with work carried on at the Storrs (Conn.) Agricultural Experiment Station, by cooperation between that station and the Dairy Division of this Bureau.

Respectfully,

A. D. Melvin,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

CONTENTS.

PROTEOLYTIC CHANGES IN THE RIPENING OF CAMEMBERT CHEESE.

INTRODUCTORY.

Until comparatively recent years the changes that take place in the ripening or curing of the different varieties of cheese were but little understood. Although the practice of cheese making has been carried on for centuries, all of our knowledge of the chemical changes involved in the ripening process and the various factors that bring about these changes has come to us within the past fifty years. The earliest record we have of any discussion of this subject from a chemical point of view was published only a century ago. In this paper the French chemist, Chaptal,[1][a] discusses the ripening of Roquefort cheese and advances certain theories to account for the changes in appearance and flavor which this cheese undergoes during its sojourn in the natural ripening caves. Biological factors were, of course, not taken into account in Chaptal's paper, for that phase of the subject was unknown until the time of Pasteur.

No scientific study of the subject, however, was made until the latter half of the nineteenth century. The attention of chemists was then directed to Roquefort cheese by a paper published by Blondeau[2] in 1864. Blondeau analyzed cheeses in different stages of ripening and found that the fat content increased from 1.85 per cent in the fresh cheese to 32.31 per cent in the cheese two months old. This enormous increase in the fat he ascribed to a synthesis of fat from protein by the mold. But a comparison of his figures for the other constituents of the cheese as well as the fat with analyses to be found in any modern book on dairy products will show their utter impossibility. This inaccurate work of Blondeau, however, served the purpose of directing the attention of other investigators to the subject, and during the next few years the changes in the fat content of cheese were studied by Brassier,[3] Sieber,[4] Jacobstahl,[5] and Von Nägeli and Loew.[6]

By this time the subject of cheese ripening had begun to arouse considerable interest among scientific investigators, and their researches were extended to other varieties of cheese. Swiss cheese and Cheddar

[a] The figures refer to list of literature at end of bulletin.

cheese in particular received considerable attention. Among those who worked with Swiss cheese were Weidmann,[7] Röse,[8] Schulze,[8] and Winterstein.[9] In our own country the Cheddar type of cheese has been made the subject of thorough chemical investigation, and in this connection reference is made to the work of Babcock and Russell[10] and that of Van Slyke and Hart.[11] The German investigators made a special study of the products of proteolysis, while those in this country studied more particularly the factors that cause the ripening.

All this work, however, deals with the "hard" cheeses. In this class of cheeses the ripening factors are the enzymes or unorganized ferments present in the fresh curd and the bacteria which occur in enormous numbers in the cheese. Such cheeses require several months for ripening, for the enzymes are present in very small amount, and the bacteria do not produce rapid proteolytic changes. In contrast to the hard cheeses we have another distinct class known as the soft cheeses. The cheeses belonging to this class differ mainly from those of the former class in that they contain a much higher percentage of water. This higher moisture content is much more favorable to the development of micro-organisms, and the ripening proceeds with greater rapidity.

PROCESSES IN THE RIPENING OF CAMEMBERT CHEESE.

The variety of soft cheese which we shall consider in this paper is the Camembert type, a soft cheese ripened mainly by a surface growth of mold. Of late years it has attained considerable importance in the cheese market, and the public is now more or less familiar with it. Although it is still imported from France in large quantities, its manufacture has been undertaken on a commercial scale in our own country with considerable success. The researches carried on by the Dairy Division of the Bureau of Animal Industry, in cooperation with the Storrs Agricultural Experiment Station, with a view to introducing the manufacture of Camembert cheese into the United States, have given very gratifying results.[12] For a description of the details of its manufacture the reader is referred to a bulletin by T. W. Issajeff.[14]

The biological factors essential to the production of Camembert cheese are the lactic-acid bacteria, which are normally present in milk, and two molds, *Penicillium camemberti* Thom[13] and *Oidium lactis*. Other molds that may be present are generally contaminations and often deleterious to the cheese. The penicillium is the mold that produces the actual ripening or digestion of the curd, while the oidium seems to be connected in some way with the flavor production. The oidium by itself or in conjunction with the lactic-acid bacteria can not ripen a cheese more than a few millimeters below the surface.

The ripening of all cheeses being essentially a hydrolysis of the paracasein or cheese curd through the agency of various enzymes, the end products or simple substances from which the complex protein molecule is built up are set free in varying amounts during the course of the ripening period. At the same time secondary reactions may occur which involve not only hydrolysis, but also oxidation, reduction, desamidation, and removal of carboxyl groups. The products of simple hydrolysis may thus undergo further changes with the formation of substances differing widely in chemical constitution from the substances from which they were derived. The successive steps of such reactions are often difficult to follow, and it is sometimes impossible to ascertain precisely what the mother substance is. In most cases, however, the secondary changes involve but one step, the removal of a certain radical from the original hydrolytic product. These changes are caused for the most part by bacteria, and in a cheese where the ripening is produced almost entirely by other agencies they are of minor importance. Where they do occur they result not from the direct action of an enzyme acting outside the cell wall of the organism, but rather from activities dependent upon the life history and metabolism of the organism. For this reason the center of a hard cheese shows the same flora and the same chemical composition as the portion near the rind. With cheeses of the Camembert-Brie type, however, there is a marked difference in this respect. The actual ripening here is caused by the proteolytic enzyme of the mold. This enzyme is secreted by the mold growing on the surface of the cheese and diffuses toward the center, digesting the curd through which it passes until the cheese is ripe. The fact that the ripening begins at the surface and proceeds toward the center indicates that the enzyme is produced in the mycelium of the mold. The progress of the ripening is very easy to follow, for the texture and color of the ripened portion are quite different from those of the unripened curd, and there is always a sharp line of demarcation between the two. The mycelium of the mold does not penetrate more than a few millimeters into the cheese, forming a sort of rind which is removed when the cheese is eaten.

The fresh Camembert cheese differs from the fresh curd of other whole-milk cheeses mainly in the greater amount of whey it contains. This slightly increases the relative amount of the other proteins, lactalbumin, whey protein, and lactoglobulin, as well as that of the nonnitrogenous constituents—lactose, citric acid, and inorganic salts. The amount of butterfat in the cheese varies merely with the richness of the milk. Paracasein, however, is the only protein present in any considerable amount, and the end products of the ripe cheese may be considered as derived from it. Moreover,

the other three proteins mentioned above yield the same primary disintegration products, and a distinction as to the origin of the latter can not be made.

The proteolytic changes which constitute the ripening of Camembert cheese consist, therefore, in the changes which this paracasein undergoes through the action of proteolytic enzymes. As has already been mentioned, the principal factor is the enzyme secreted by the Camembert penicillium. Other enzymes are present, however, and a brief discussion of these will follow. No appreciable proteolysis occurs until after the cheese is nearly 2 weeks old. But in the meantime certain changes take place in the character and solubility of the curd. These changes have been studied by Bosworth.[15] They consist mainly in the liberation of paracasein from combination with calcium, due to the formation of lactic acid by lactic acid bacteria. At the same time the paracasein is converted into a form completely soluble in 5 per cent salt solution, and later it becomes insoluble again. As these are not, strictly speaking, proteolytic changes, a detailed discussion will not be given here.

ENZYMES IN THE CHEESE.

With the exception of the enzyme secreted by the mold and of a smaller variety of bacteria, Camembert contains the same ferments that are present in other cheeses. Like the hard cheeses, it contains the milk enzyme galactase, the rennet enzyme chymosin added in the curdling process, and lactic acid bacteria. In the case of the Cheddar type of cheese the action of these three factors has been studied in detail. Babcock and Russell found that galactase and rennet (pepsin) were important agents in the ripening of this variety of cheese. According to Van Slyke and Hart,[11] the rennet alone is capable of ripening a cheese. In their experiments the galactase was first destroyed by heat and then chloroform added to prevent the development of bacteria, yet the ripening went on, though not as rapidly as in the normal cheese, and the character of the chemical products was somewhat different. Thus there was a predominance of paranuclein, caseoses, and peptones, and an abnormally small amount of amino-acids. The entire absence of ammonia was very striking. The function of the bacteria in this variety of cheese has been studied by Rogers.[16] He came to the conclusion that the enzymes produced by the bacteria were responsible for most of the digestion beyond the peptone stage, and consequently the characteristic flavors.

In the short time required for the ripening of Camembert cheese the rennet, galactase, and lactic acid bacteria produce no appreciable digestion. This conclusion was reached by Bosworth, and the experience of the writer confirms it. Even the hard cheeses which are

ripened entirely by these agents undergo very little change during the first month. A Camembert cheese, however, should be ripe at the end of a month, and at the same time should contain a greater amount of primary digestion products. This ripening must be due almost entirely to the mold enzyme, for the interior curd, which has not yet been reached by this enzyme, but contains all of the other ferments, shows little evidence of digestion. If the unripened curd in the center of a Camembert cheese three or four weeks old be subjected to chemical analysis it will be found that the paracasein is scarcely altered except for the fact that it is liberated from combination with calcium. The galactase can not play more than a very subordinate rôle, as is shown by the fact that the cheese ripens normally when made from milk which has been pasteurized at a temperature sufficiently high to impair greatly, if not destroy, the activity of this enzyme. Likewise the rennet can not be of more than minor importance as a ripening factor. Recent investigations have shown that chymosin or rennet enzyme is identical with pepsin. But, as will be seen later, the ripening of Camembert cheese bears no resemblance to a peptic digestion. The rennet should show its greatest activity in the interior curd, which is quite strongly acid. But owing to the short duration of the ripening period and the small amount of rennet present, the proteolytic action of the latter is practically negligible. Aside from the hydrolysis of the casein into paracasein and whey protein, its action is inappreciable. The unripened curd shows no evidence of peptic digestion.

The lactic acid bacteria which constitute nine-tenths of the bacterial flora of the cheese serve the purpose of converting the milk sugar into lactic acid, thus producing conditions unfavorable to the development of other bacteria. They are probably responsible for the peculiar flavor which is characteristic of the acid curd in the interior of a Camembert cheese. Their proteolytic action is otherwise hardly noticeable. Experiments in which sterile curd was inoculated with these organisms show that the amount of diffusible nitrogen increases only very slightly at the end of a month, even in the presence of calcium carbonate, which neutralizes the acid.

We are safe in assuming, therefore, that these three proteolytic factors—the galactase, the rennet, and the lactic acid bacteria—have very little to do with the actual ripening of the cheese, this being essentially the work of the enzyme from the mold.

As has already been pointed out, the mold of Camembert cheese (*Penicillium camemberti*) secretes a powerful proteolytic enzyme, which is undoubtedly the most potent factor in the ripening of this cheese. The fact that the ripening begins at the surface and proceeds toward the center indicates that the enzyme is produced in the mycelium of the mold and diffuses inward. The diffusibility of this enzyme

is also shown by the fact that synthetic culture media upon which this mold has grown for some time have a marked proteolytic activity. Experiments are now being instituted to determine the exact nature of this enzyme and the extent to which it will hydrolyze certain proteins. The results thus far obtained seem to indicate that it is of the nature of erepsin. It attacks casein and peptone readily, but is without action upon fibrin and coagulated egg albumin.

Vines[17] has shown that erepsin is very widely distributed in the vegetable kingdom. This vegetable "ereptase," as he calls it, differs from animal erepsin in that it is most active in the presence of the natural acid of the plant. The addition of other acids, or of an alkali, greatly impairs its activity. As long as the acidity is due entirely to acid phosphates, the activity of this ereptase is very pronounced, but the presence of free acid in the medium is inhibitory. It is readily seen, therefore, that a fresh Camembert cheese offers very favorable conditions for the action of ereptase. In the first place, casein and paracasein are readily attacked by this enzyme. In the second place, the lactic acid produced by the bacteria does not accumulate but combines with the calcium phosphate, forming calcium lactate and mono-calcium phosphate. The acidity of the cheese is due to the presence of the latter salt.

In this connection, however, it must be remembered that the same results are not necessarily obtained with enzymes in the presence of an antiseptic as with organisms *in vivo*. In the case of Camembert cheese where the proteolysis is effected by diffusion of the enzyme rather than by diffusion of the substratum, the conditions more nearly approach those met with in an artificial digestion experiment. Nevertheless there are certain striking differences which will be pointed out later. A discussion of the enzymes obtained from a pure culture of this organism will be reserved for a future paper.

PROTEOLYSIS OF CASEIN.

When casein or any other protein is boiled with strong acid or alkali, a decomposition takes place with the production of simpler substances. These simple substances resulting from such decomposition have of late years been studied by a number of investigators. A similar decomposition occurs when the protein is acted upon by proteolytic enzymes. These enzymes also have the power of transforming casein into bodies of less molecular complexity. The changes are of a hydrolytic nature, the original molecule being broken successively at different places and a molecule of water entering at the point of cleavage. The extent to which the protein is hydrolyzed, and consequently the nature of the resulting products, depends upon the enzyme. Pepsin, obtained from the gastric juice, does not carry the proteolysis beyond the peptone stage, while tryp-

sin, obtained from the pancreas, breaks up the protein into crystalline end products. Erepsin, on the other hand, does not attack native proteins, with the exception of casein, but acts readily upon proteoses and peptones. The enzyme isolated by the writer from Camembert mold resembles erepsin in this respect.

NITROGENOUS CONSTITUENTS OF THE CHEESE.

The ripening of Camembert cheese being a proteolysis, certain digestion products may be expected to occur in the ripened cheese. As casein, or rather paracasein, forms the main bulk of the proteins in the curd, it would therefore undergo the same changes that occur when casein is digested artificially with an enzyme, though the proteolysis is never allowed to go on as far in a cheese as is usually done in an artificial digestion experiment. The products may be grouped roughly into the following classes: Caseoses, peptones, polypeptids, amino-acids, and ammonia. Methods for the separation of these groups of substances from cheese have already been elaborated by Van Slyke and Hart.[11] They consist in extracting the cheese with water, and determining the nitrogen in the precipitates obtained by the addition of various reagents to aliquot portions of this extract.

In the subsequent pages of this article the separation of the individual members of these groups will be discussed. The cheeses used for this work were made at the Storrs Experiment Station, and were pronounced by experts to be equal in texture and appearance to the imported brands. Both texture and flavor showed them to be excellent cheeses of the Camembert type.

The analysis of a ripe cheese by the method of Van Slyke and Hart showed the nitrogenous constituents to be present in the following amounts:

Nitrogen as—	Per cent of cheese.
Total nitrogen	2.47
Water—soluble	1.79
Precipitated by hydrochloric acid	.40
Caseoses	.10
Peptones	.28
Amino-acids	.82
Salt—soluble (paracasein)	.15
Ammonia	.19

The polypeptids are included under the peptones and amino-acids. This represents the analysis of an individual cheese. Some variations occur with different samples, but the differences are only slight with cheeses at the same stage of ripening. As none of the individual members of these groups of digestion products had ever been isolated from Camembert cheese, a determination of some of the more characteristic ones was considered advisable. Proximate analyses of the

cheese at different stages of ripening will be found in Bosworth's paper.[15] These analyses show merely the rate of formation or destruction of three broad groups of digestion products, and throw little light upon the nature of the ripening from the biochemical standpoint. The writer has attempted to determine, as far as the facilities at his command permitted, the relative amounts of the more important members occurring in these groups. They will be discussed in the same order as the groups are determined in Van Slyke and Hart's method for the analysis of cheese.

HYDROCHLORIC ACID PRECIPITATE.

When an aqueous extract of the cheese is made and acidified with hydrochloric acid, a white curdy precipitate appears, which on warming to 50° C. clots together into a gummy mass. If the digestion were of a peptic nature this precipitate should consist of paranuclein. The chief characteristics of this altered phosphoprotein are its high phosphorus content and the comparative slowness with which it is further acted upon by pepsin. On the other hand, trypsin converts casein directly into caseoses without the formation of paranuclein, and at the same time liberates the phosphorus in the form of phosphoric acid. Erepsin probably acts in the same way as trypsin in this respect. Again, the precipitate might be a coagulose or protein synthesized by the reversed action of a proteolytic enzyme. Such a coagulose is Kurajeff's plastein, formed by the action of rennet on albuminoses.

A similar precipitate has been obtained from other varieties of cheese and has usually been regarded as paranuclein. As the ripening of Camembert cheese is not a peptic digestion, it seemed unlikely that this precipitate could be paranuclein. In order to determine more precisely the nature of the precipitate, several cheeses were subjected to the following treatment.

An aqueous extract was made by stirring the macerated cheese with water in a bath maintained at a temperature of 50° C. This extraction was repeated several times, filtering off the liquid at the end of half an hour through cotton and asbestos, and adding fresh quantities of water until the filtrate was practically free from nitrogenous matter. The extract made in this way was acidified to 0.2 per cent with hydrochloric acid, whereupon a white curdy precipitate resulted. The precipitate was washed thoroughly with acidulated, then with distilled, water, and finally dried in a desiccator and extracted with ether to remove adhering fat.

Upon testing the solubilities of this precipitate, it was found that a small part of it dissolved in 5 per cent sodium chlorid solution, while the greater part was soluble in 50 per cent alcohol. The smaller fraction was studied first. It dissolved in alkalies, was reprecipitated by acids, excess of which dissolved the precipitate.

The substance was readily attacked by trypsin, dissolving completely in twenty-four hours, and giving a solution from which no precipitate was obtained by saturation with ammonium sulphate. A sample dried at 110° C. gave the following analysis, based upon the ash-free substance. Alongside it are given Röse's analysis of paracasein and Chittenden's[18] analysis of paranuclein, with the phosphorus as found by Jackson.

	Substance from cheese.	Paracasein (Röse).	Paranuclein (Chittenden).
Carbon	53.63	53.94	51.29
Hydrogen	7.08	7.14	7.26
Nitrogen	15.10	15.14	15.23
Sulphur	.98	1.01	.68
Phosphorus	.50	.....	2.75
Ash	.82	.....	12.43

A comparison of these analyses shows that the substance is too low in phosphorus to be paranuclein. The analysis agrees fairly well with that of paracasein, and it can be regarded as paracasein from which a part of the phosphorus has been liberated by enzyme action. Paracasein probably contains about the same amount of phosphorus as casein itself, which, according to Hammarstein, is 0.85 per cent.

The alcohol-soluble part of the precipitate was dissolved in 50 per cent alcohol, filtered and poured into water. A gummy precipitate was formed. It was dried at 110° C., extracted with ether, and analyzed. On fusing the substance with potash and niter for the sulphur determination, a strong odor of skatol was emitted, stronger than that obtained with casein. This indicates the presence of the tryptophane group. The ready solubility in alcohol and insolubility in water are properties characteristic of caseoglutin, a substance discovered in Swiss cheese by Weidmann. The analysis, together with Röse's analysis of caseoglutin, follows:

	Substance from cheese.	Caseoglutin (Röse).
Carbon	54.34	54.4
Hydrogen	7.30	7.34
Nitrogen	15.37	15.29
Sulphur	.95	.95
Phosphorus	.06	.....
Ash	.28	.....

Both the analysis and the properties of this substance agree with those of caseoglutin.

COAGULABLE PROTEINS.

Winterstein found in Swiss cheese a small amount of a substance which was precipitated from acid solution by boiling. This substance he calls tyroalbumin. Its exact nature has not yet been determined.

Several attempts were made to find this substance in Camembert cheese, but so far they have resulted in failure. On heating the filtrate from the caseoglutin to boiling, both in acid and in neutral solution, no precipitate was obtained.

CASEOSES.

These, together with the peptones, are the intermediate disintegration of casein by ordinary proteolytic enzymes. They can not be regarded as homogeneous substances, as they represent transition products formed by the loss of varying numbers of amino-acid molecules from the original protein. They can, however, be separated into groups according to their solubilities. A method for the separation of albumoses by fractional precipitation with ammonium sulphate was elaborated by Pick.[19] If nitrogen determinations are to be made, the ammonium sulphate must be removed by dialysis, a long and tedious operation. To obviate this difficulty Zunz[20] used zinc sulphate and found that the precipitation limits were quite as sharply defined. As the elementary analyses of these groups of caseoses have very little value, and the peptones were to be separated from the filtrate, the method of Pick was used in this work. These saturation limits can not, however, be regarded as reliable indexes of individuality.

The caseoses of the cheese were separated into the four fractions described by Pick. They are designated as follows: Protocaseose, by half saturation of the neutral solution with ammonium sulphate; deuterocaseose A, by two-thirds saturation; deuterocaseose B, by complete saturation; and deuterocaseose C, by acidifying the filtrate from B. In the early stages of ripening, the protocaseose predominates. In the ripened cheese, however, protocaseose and deutero B are present in about equal amounts, and together form about three-fourths of all the caseoses. A distinction will be noticed here from the albumose formation observed by Zunz in peptic digestion. According to Zunz, after deutero B has reached its maximum, deutero A predominates, and finally deutero C.

In purifying the different fractions, the method of Haslam[21] was followed out, viz, rubbing the precipitate in a mortar with ammonium sulphate solution of the same concentration as the filtrate. Several reprecipitations were made before the product was finally freed from ammonium sulphate by repeated precipitation with alcohol.

The first fraction should contain, besides protocaseose, heteroalbumose if this substance were present in the cheese. Heteroalbumose could not be derived from casein. Traces were found, however, but they probably came from albumin. Upon subjecting the carefully purified protocaseose to dialysis, a slight residue was left

which would not diffuse. The amount was too small for chemical examination, but it was probably heteroalbumose. All the fractions gave the biuret reaction, and all except deutero C gave the Millon reaction. The intensity of the lead sulphid reaction seemed to diminish progressively, until with deutero C it was just perceptible.

PEPTONES.

The filtrate from the caseoses was nearly neutralized with ammonia and treated with a saturated solution of ferric ammonium sulphate. A gelatinous brown precipitate resulted. This corresponds to the alpha and beta peptones of Siegfried.[22] The precipitate was filtered off, washed with a saturated solution of iron alum, and decomposed by barium hydrate. After filtering off the ferric hydroxid and barium sulphate, a current of air was drawn through the alkaline solution until the ammonia was expelled. The barium was then removed by sulphuric acid, and the solution concentrated under diminished pressure and poured into a large volume of alcohol. A precipitate was obtained which is analogous to Winterstein's alpha peptone. The filtrate still contained peptone, as was shown by an intense biuret reaction. Further addition of alcohol gave no precipitate. The solution must therefore contain an alcohol-soluble peptone—Winterstein's beta peptone. It was reprecipitated, after distilling off the alcohol, by phosphotungstic acid, the precipitate decomposed by barium hydroxid, and the barium removed by sulphuric acid, carefully avoiding an excess. The solution was then evaporated to dryness. The resulting beta peptone may have contained slight admixtures of polypeptids, but further purification was not attempted.

The alpha peptone gave no Millon reaction and a strong furfurol reaction. Beta peptone, on the other hand, gave a strong Millon reaction, but no furfurol reaction. Both gave the characteristic red biuret reaction, xanthoproteic reaction, and a slight lead sulphid reaction. Dried at 105° C., alpha gave 15.10 and beta 14.80 per cent nitrogen. In this case the analytical figures have very little value, as the substances are hygroscopic, and on drying continue to lose water until a temperature is reached which causes a slight decomposition. The two peptones were present in about equal amount and together comprised about 1.6 per cent of the cheese. They had the bitter taste characteristic of peptones.

POLYPEPTIDS.

After removal of the caseoses and peptones by means of lead acetate, carefully avoiding an excess of the reagent, the cheese extract still gave a biuret reaction. The polypeptids are the intermediate products between peptones and amino-acids. Some of them give a biuret

reaction, and their presence is probably the explanation of this phenomenon. Some are precipitated by lead acetate, while others remain in solution. Fischer and Abderhalden obtained by the tryptic digestion of casein a polypeptid, which on hydrolysis yielded alpha-pyrrolidin-carboxylic acid and phenylalanin. These two acids were not found in the free state, and their absence has been regarded as characteristic of tryptic digestion. Whether or not erepsin decomposes this polypeptid is not yet known. It is very probable that other polypeptids exist, temporarily at least, as transition products from the peptones to the amino-acids. Many of them would be destroyed by the action of the mold, while others would be more resistant. Abderhalden[23] found that *Aspergillus niger* grows readily on glycylglycin and dileucylglycylglycin, two polypeptids that are not attacked by trypsin. Winterstein regards his alpha peptone as similar in many respects to Fischer's polypeptid. This, however, would seem improbable, in view of the fact that he has demonstrated phenylalanin and prolin in the cheese. No satisfactory method has yet been found for separating the polypeptids as a group, and the amount present in a cheese can only be a matter of conjecture. The polypeptids will be made the subject of future study in this connection.

DIAMINO-ACIDS.

The next group of substances to be studied was the diamino-acids, or hexone bases. The three hexone bases, along with ammonia, can be determined quantitatively, and for that reason they have received more attention from investigators studying the disintegration products of the proteins than have the monoamino-acids. They can be expressed in definite figures, whereas the other disintegration products have to be expressed in minimal values. They have already been found in cheese—in Swiss cheese by Winterstein and Thöny, and in Cheddar cheese by Van Slyke and Hart. Owing to the softer consistency of Camembert cheese a somewhat different method of procedure was adopted in making the extraction.

Three kilograms of the thoroughly ripened cheese made at the Storrs Experiment Station were ground in a mortar and extracted six times with warm water, according to the usual method of analysis, until the volume of the liquid was about six liters. The greater part of the fat rose to the surface and could easily be skimmed off. It was washed by stirring thoroughly with cold water and the filtrate mixed with the cheese extract. The remainder of the fat was found to be precipitated almost quantitatively with the proteins, and thus the necessity of extracting the original cheese with ether was obviated. The extract was filtered through cotton and through asbestos, then acidified with sulphuric acid and warmed until the caseoglutin had

settled out, whereupon the liquid was filtered again. The solution was now concentrated at a low temperature until the volume was about two liters. About three volumes of alcohol were added to precipitate the greater part of the caseoses and peptones. After filtering off this precipitate the alcohol was distilled off under diminished pressure, and tannic acid added to precipitate the rest of the caseoses and peptones. The excess of tannic acid was removed by lead acetate and the lead by sulphuric acid. The resulting solution still gave a biuret reaction. It contained, besides traces of secondary disintegration products and polypeptids, the hexone bases, amino-acids, and ammonium salts, together with the sodium chlorid present in the cheese.

The hexone bases were precipitated by a 50 per cent solution of phosphotungstic acid in the presence of 5 per cent sulphuric acid. A large amount of this reagent had to be added before the precipitation was complete, and a voluminous white precipitate was obtained. After standing several days it was filtered off and washed with 5 per cent sulphuric acid containing a little phosphotungstic acid. The washing was a long and tedious operation. It was found necessary to remove the precipitate each time from the funnel and grind it with the sulphuric acid in a mortar. This was repeated until all of the sodium salts had been removed. .

For the separation of the bases Kossel's[24] older method was used, after removal of the phosphotungstic and sulphuric acids by barium hydroxid and passing in a current of air to expel the ammonia. The excess of barium was removed by carbon dioxid, and mercuric chlorid added to precipitate the histidin. This precipitate was allowed to stand several days, then filtered and washed again. It was suspended in water and decomposed by hydrogen sulphid after slightly acidifying with sulphuric acid. The filtrate from the mercuric sulphid was boiled with charcoal until practically colorless, and precipitated with silver nitrate and ammonia. The arginin was precipitated by saturating the solution with barium hydroxid, and adding silver nitrate until a drop of the solution gave a brown color on the addition of silver nitrate. The arginin silver was decomposed by hydrochloric acid and hydrogen sulphid, filtered, boiled with charcoal, and evaporated to crystallization. The filtrate from the arginin was freed from barium and silver by means of sulphuric acid and hydrogen sulphid, and an alcoholic solution of picric acid added. The lysin picrate did not crystallize readily, but after several crystallizations the characteristic yellow needles were obtained. The bases were found in the following amounts: Histidin, 1.1 grams; arginin, 0.6 gram; and lysin, 1.9 grams. Histidin was analyzed in the form of the silver salt, arginin as the chlorid, and lysin as the picrate. The

free histidin gave an intense red color with diazobenzenesulphanilic acid. The analyses of the bases are given below:

Histidin silver, $C_6H_7N_3O_2Ag_2H_2O$.

(Beta-imidoazol-alpha-aminopropionic acid.)

	Calculated.	Found.
Argentum	55.81	55.60
Nitrogen	10.85	10.78

Arginin hydrochlorid, $C_6H_{14}N_4O_2HCl$.

(Delta-guanidine-alpha-aminovalerianic acid.)

	Calculated.	Found.
Chlorin	16.87	16.70
Nitrogen	22.60	22.50

Lysin picrate, $C_6H_{14}N_2O_2C_6H_3N_3O_7$.

(Diaminocaproic acid.)

	Calculated.	Found.
Nitrogen	18.66	18.50
Carbon	38.40	38.53
Hydrogen	4.53	4.54

The other bases were present in so small amount (about 0.5 gram) that no attempt was made to isolate them. A noteworthy fact is that arginin, which was not found at all in Swiss cheese, is present here. It is possible that some of it is further hydrolyzed into guanidin and aminovalerianic acid or into urea and ornithin (diaminovalerianic acid). The filtrate from the histidin, arginin, and lysin had a very faint but characteristic odor of tetramethylenediamin. This substance would result from the liberation of carbon dioxid from ornithin, one of the cleavage products of arginin. It could not have been present, however, in more than traces. An attempt was made to separate guanidin in the form of the gold salt, but no crystals could be obtained. The small amount of bases remaining in the lysin fraction indicates that the occurrence of secondary reactions in this group is very slight.

MONOAMINO-ACIDS.

A complete separation of all the amino-acids remaining in solution after removal of the intermediate disintegration products and hexone bases can be accomplished only by Fischer's method of distilling the ethyl esters in vacuo. This necessitates delicate and costly apparatus

which the writer did not have at his command. Certain of the amino-acids, however, can be separated almost quantitatively from the mixture without resorting to the method of esterification. Among these are glutaminic acid, tyrosin, and leucin.

Another lot of cheese (3 kilograms) was treated in the manner described above to remove the caseoglutin, caseoses, and peptones. The residue was evaporated to a small bulk, saturated with hydrochloric-acid gas, and kept at zero for several days. Crystals were deposited on the walls of the flask, and a pulverulent precipitate separated out on the bottom. These were found to consist of glutaminic acid, hydrochlorid, and sodium chlorid. They were transferred to a Büchner funnel and washed with concentrated hydrochloric acid, then dissolved in water. The solution was neutralized with caustic soda and boiled with freshly precipitated copper hydroxid. A blue precipitate was formed. It was filtered and washed, and then suspended in water slightly acidified, and decomposed by hydrogen sulphid. The free acid thus obtained was again saturated with hydrochloric-acid gas and allowed to crystallize as before. The colorless crystals thus obtained were decomposed by the calculated amount of caustic soda (30 c. c. N–NaOH), and the free acid crystallized out. About 5 grams of crystals were obtained. Analysis gave the following figures:

Glutaminic acid, $C_5H_9NO_4$.

(Aminoglutaric acid.)

	Calculated.	Found.
Carbon	40.82	40.62
Hydrogen	6.13	6.15
Nitrogen	9.52	9.53

The filtrate from the glutaminic acid was treated with lead carbonate to remove the bulk of the hydrochloric acid, and the lead remaining in solution was removed by sulphuric acid. After filtering and neutralizing, the solution was evaporated to incipient crystallization. The first crop of crystals should contain tyrosin and traces of leucin, and the second leucin with traces of tyrosin. The two constituents of each fraction were separated by treatment with glacial acetic acid. The leucin purified in this way gave no color with Millon's reagent. The tyrosin was tested for sulphur by fusing a portion of it with sodium carbonate and adding sodium nitroprussid to the aqueous solution. No coloration was obtained, indicating the absence of cystin. About 8 grams of tyrosin and 14 grams of leucin were obtained. It must be borne in mind that while the greater part of the glutaminic acid and leucin can be isolated in this way, the amounts do not represent strictly quantitative results, for a further yield is

invariably obtained from the higher boiling fractions of the ethyl esters. Following are the analyses of the tyrosin and leucin:

Tyrosin, $C_9H_{11}NO_3$.

(P-hydroxyphenyl-alpha-aminopropionic acid.)

	Calculated.	Found.
Carbon	59.66	59.53
Hydrogen	6.07	6.00
Nitrogen	7.73	7.69

Leucin, $C_6H_{13}NO_2$.

(Alpha-aminoisobutylacetic acid.)

	Calculated.	Found.	
		First crop.	Second crop.
Carbon	53.96	54.99	54.82
Hydrogen	9.92	9.89	9.62
Nitrogen	10.68	10.53	10.76

The tyrosin gave the characteristic color reactions, viz, a red color and precipitate with Millon's reagent, a bright red coloration with diazobenzenesulphanilic acid, and a yellow precipitate of nitrotyrosin nitrate with nitric acid. Under the microscope it showed the characteristic wavy needles.

The leucin was also characteristic under the microscope. Heated on a platinum foil it sublimed completely, emitting an odor like that of some of the higher alkylamins.

On further concentrating the filtrate from the leucin, a few needle-shaped crystals were obtained having a sour taste. They did not melt at 225° C. Not enough of the substance was obtained for analysis, but it was probably aspartic acid.

As has already been mentioned, the remainder of the amino-acids can not be separated without resorting to Fischer's method of distillation. Phenylalanin and tryptophan, however, can be detected qualitatively. On evaporating the solution to dryness and treating the residue with sulphuric acid and potassium dichromate, the writer felt confident that he detected the odor of phenylacetaldehyde, notwithstanding the presence of other aldehydes formed by the oxidation with dichromate. This would indicate the presence of phenylalanin, but from this test alone it would be impossible to say with certainty whether phenylalanin was really present or not.

A striking fact is that none of the cheeses examined responded to the tryptophan reaction with acetic acid and bromin water. Aque-

ous extracts of cheeses in all stages of ripening were examined, but in all cases they failed to give any coloration with this reagent. When the mold was grown upon milk the tryptophan reaction was likewise negative, although the casein was broken down into amino-acids, among which leucin and tyrosin were identified. On the other hand, the enzyme preparation from the same mold readily digests milk in the presence of toluol, and the tryptophan reaction is invariably positive. If tryptophan were liberated in the cheese, it might undergo further decomposition as a result of bacterial action. In this case the end products would be indol and skatol—characteristic products of putrefaction. Both of these substances would have given a coloration with the tryptophan reagent, and must therefore have been absent.

AMMONIA.

A normal cheese contains from 0.20 to 0.25 per cent of ammonia. The greater part of this is in combination with acid radicals, for while the ripened cheese is alkaline to litmus it is still acid to phenolphthalein. Some of the more highly flavored specimens, however, have a distinct odor of ammonia near the rind. The formation of ammonia does not begin until after the second week, then the amount steadily increases until the cheese is ripe. As long as the ripened portion does not extend more than a few millimeters below the mycelium of the mold no ammonia, either free or in combination, can be detected. Cheeses with a very strong flavor contain, as a rule, more ammonia than the milder ones. In cheeses that are overripe the presence of free ammonia can usually be detected by its odor. For the determination of ammonia, the writer has found Folin's method more satisfactory than the regular method of distilling the tannin filtrate with magnesium oxid. It can be used in the presence of proteins without effecting any decomposition.

ABSENCE OF PUTREFACTIVE PRODUCTS.

While the ripened cheese possesses a peculiar odor, which to some people is quite disagreeable, it does not resemble that of the typical putrefactive products, nearly all of which are characterized by a foul odor. Among the substances belonging to this class are indol, skatol, mercaptan, hydrogen sulphid, and phenols. Qualitative tests were made repeatedly for all of these substances on different samples of cheese, but in all cases they were negative, except in those cheeses that had gone past the usual ripening. After the ripening is complete putrefaction may set in if the cheese does not receive proper care. In those cases where some of the putrefactive products were found the cheeses were otherwise unfit for eating, as was evidenced by a very

disagreeable odor and taste. Nearly all of these substances are found in Limburg cheese, but they do not occur in good Camembert cheese. The failure to find typical putrefactive products, together with the fact that the lysin fraction of the diamino-acids contained only traces of diamins, indicates that whatever the action of the bacteria in the cheese may be, they do not cause secondary reactions of this nature to any extent.

SUMMARY.

The following substances have been isolated from Camembert cheese: Caseoglutin, protocaseose, deuterocaseoses A, B, and C, alpha and beta peptones, histidin, arginin, lysin, glutaminic acid, tyrosin, and leucin.

Among those substances which the writer failed to find are paranuclein, tryptophan, indol, skatol, mercaptan, hydrogen sulphid, and phenols.

The ripening of Camembert cheese can not be a peptic digestion, as is shown by the following facts:

1. Paranuclein, the characteristic product of peptic digestion of casein, is absent.

2. The greater part of the phosphorus is liberated and appears as acid calcium phosphate. According to Plimmer and Bayliss,[25] pepsin acts slowly and incompletely, only 70 per cent of the phosphorus being liberated from casein in 149 days, and that mostly in the organic form.

3. Amino-acids and ammonia are present in considerable amount.

The ripening resembles ereptic digestion in many respects, as follows:

1. The reaction of the cheese before ripening, i. e., acidity caused by acid phosphates, is most favorable to the activity of ereptase.

2. The digestion proceeds beyond the peptone stage, with the formation of amino-acids and ammonia.

3. A separate study of the enzyme from the Camembert mold shows that this enzyme is a vegetable ereptase.

The absence of tryptophan, which is ordinarily liberated in ereptic digestion, is striking.

The presence of caseoglutin, the remarkable albumose-like body, is also noteworthy. This substance has not, to the writer's knowledge, been observed in digestions with pure enzymes.

ACKNOWLEDGMENTS.

In conclusion, the writer acknowledges his indebtedness to Prof. L. B. Mendel, of Yale University, and to Dr. B. B. Turner, formerly of the Storrs Experiment Station, for many helpful suggestions in carrying out this work.

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